

codon assignments. Although the suggestion is certainly strong here, the alternative presented by Sonneborn has not yet been eliminated. To recapitulate the Sonneborn argument, selection pressure will bring about a code in which mutations produce a minimal amount of lethality. This means that (1) "nonsense" codons will be eliminated or minimized (some codons may have to serve a punctuation function), (2) the fraction of mutations which involve a codon change *not* leading to an amino acid change will be optimized, as will (3) the fraction of mutations involving codon changes leading to replacement of a given amino acid by a "functionally related" amino acid. I feel that while the end result of the Sonneborn scheme is plausible, there is no plausible mechanism by which the code could have evolved to this degree of perfection (without some underlying amino acid-nucleotide interactions). A scheme such as Sonneborn's would involve countless evolutionary trials and errors, and I feel that the possibilities for evolving into "blind alleys" (forms of the code having a far lower degree of order) so far outnumber the possibilities for evolving an optimal code (the one observed) that the latter could never have evolved in this way. However, it must be admitted that without a proper analysis of the Sonneborn model—such as a computer study—this counterargument remains feeble. Thus the question is not completely resolved at this time.

One thing is clear from the above, however. Extensions of amino acid chromatography in which one of the phases resembles more closely nucleic acid components may well turn out to be useful in elucidating possible amino acid-nucleotide interactions.

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PENICILLIN: ITS BASIC SITE OF ACTION AS AN INHIBITOR OF A PEPTIDE CROSS-LINKING REACTION IN CELL WALL MUCOPEPTIDE SYNTHESIS*

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Communicated by Rollin D. Hotchkiss, May 17, 1965

It has been known for some time that penicillin interferes with bacterial cell wall mucopeptide synthesis.¹⁻³ When a penicillin-sensitive cell grows in the presence of penicillin, the integrity of the cell wall is lost and the cell either ruptures or its membrane is damaged beyond repair.^{4, 5}

Although this general picture of the over-all effect of penicillin on growing cells

has been well known, many specific questions concerning its action remain to be understood, for example: (1) What specific reaction required for mucopeptide synthesis is inhibited by penicillin? (2) What normal cell wall metabolite might penicillin resemble and therefore antagonize by virtue of this chemical similarity? (3) What causes loss of integrity of the cell wall if penicillin merely inhibits new synthesis? (4) Why is continued growth necessary for penicillin to be bactericidal? (5) Why is penicillin less effective than vancomycin or bacitracin in inhibiting mucopeptide synthesis? This report provides new evidence and offers an hypothesis which helps to clarify these problems.

Cell Wall Mucopeptide Structure and the Importance of the Peptide Cross-Linkage to the Integrity of the Cell.—The general structure of the mucopeptides is shown in Figure 1. It can be seen that an outstanding structural feature of mucopeptide is that the polysaccharide chains are covalently linked through peptide bridges.^{6, 7} These chains of polysaccharide are composed of alternating units of the sugars, N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (3-O-D-carboxy ethyl N-acetyl glucosamine) (MurNAc). The carboxyl group of muramic acid is linked to the short peptides which are characteristic of mucopeptide. Neighboring polysaccharide chains are covalently cross-linked via the peptides to form one giant bag-shaped molecule (estimated mol wt = 50 billion).⁶

Obviously, the unique strength of mucopeptide resides in its cross-linked netlike structure. A single net or "sheet" probably exists in Gram negative bacteria such as *Escherichia coli* which have walls containing only 10–15 per cent of mucopeptide. Gram positive cells, in contrast, have 50 per cent or more of their wall as mucopeptide. The result is a correspondingly thicker and stronger structure which may be visualized as a number of sheets of mucopeptide cross-linked to one another. Anyone who has compared resistance of bacterial cells to rupture by physical means can attest to the strength of *Staphylococcus aureus* cells relative to those of *E. coli*.

Cross-linking of mucopeptide chains was first suggested by the end group analyses

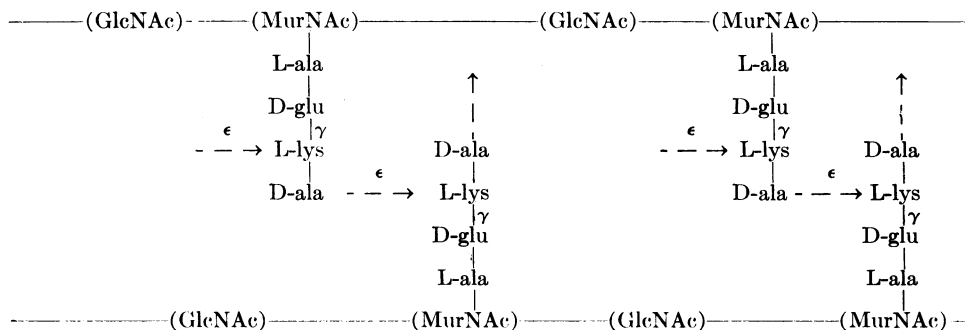


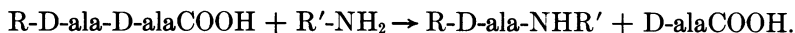
FIG. 1.—General structure for cell wall mucopeptide. In the mucopeptide of certain bacteria other diaminoacids substitute for L-lysine such as *meso* diaminopimelic acid, L,L diaminopimelic acid, α,γ diaminobutyric acid, and ornithine. Cross-linking occurs between the carboxyl group of the D-alanine of one tetrapeptide and the free amino group of the diaminoacid of a neighboring tetrapeptide. Additional amino acids may be involved in the cross-linking bridge in some instances, e.g., in *S. aureus* H, a pentapeptide of glycine is attached to the epsilon amino group of lysine and the cross-link occurs between D-alanine and the terminal free amino group of glycine. The polysaccharide chains pictured may be cross-linked in such a way as to form a single sheet or in a more random fashion to form a thicker polymer. Dotted arrows indicate the positions of the cross-links and denote the fact that the cross-linking bridges may contain one or more additional amino acids.

of Salton⁸ who showed that few of the carboxyl or amino groups in mucopeptides were free terminal groups. The bacterial cell wall mucopeptide must be extensively cross-linked in this manner to preserve the integrity of the cell. This can be inferred from the well-established observation that enzymes which hydrolyze specific peptide bonds in the mucopeptide^{9, 10} lyse cells almost as readily as lysozyme and related enzymes⁷ which attack only the polysaccharide portion of the molecule.

The Hypothesis that Penicillin Inhibits the Cross-Linking Reaction in Mucopeptide Synthesis.—The familiar precursor of mucopeptide in *S. aureus*—uridine-5'-pyrophosphoryl N-acetyl muramyl-L-alanyl- γ -D-glutamyl-L-lysyl-D-alanyl-D-alanine (UDP-acetyl muramyl pentapeptide)—contains 2 residues of D-alanine, whereas the final mucopeptide units contain only one D-alanine. This is presumably a general mucopeptide product-precursor relationship for the following reason: Oxamycin has been shown to inhibit mucopeptide synthesis in staphylococci and streptococci by competing with D-alanine for both D-alanyl-D-alanine synthetase and alanine racemase, thereby depriving the cell of the D-alanyl-D-alanine needed to make the mucopeptide precursor—UDP-acetyl muramyl pentapeptide.^{11, 12} Most bacteria are sensitive to oxamycin. This fact suggests that most bacteria utilize a precursor with 2 D-alanines, whereas analyses of walls of a variety of bacteria indicate (with rare exceptions) approximately one D-alanine in the mucopeptide per repeating unit.

Recently, studies have been made of the biosynthesis of staphylococcal mucopeptide by a cell-free membrane particle system.^{13, 14} It has been observed that utilization of UDP-acetyl muramyl pentapeptide produces membrane-bound mucopeptide which still contains 2 D-alanine residues per repeating unit.^{15–17} Penicillin does not inhibit this reaction,^{13, 14, 17} nor does penicillin completely inhibit mucopeptide synthesis in intact cells. In fact, under certain conditions huge doses of penicillin (relative to the minimum inhibiting dose) only reduce the rate of mucopeptide synthesis by 50 per cent.

In seeking an explanation for this during the past year, we came to the postulate that the final or cross-linking step in mucopeptide synthesis in *S. aureus*, and indeed in all bacteria, might proceed by a transpeptidation reaction with the release of the extra D-alanine. This is illustrated by the equation:



(R and R' are the portions of mucopeptide repeating units not directly involved in the cross-linking transpeptidation reaction.) The bond energy of the terminal D-alanine would make possible a two-component transpeptidation reaction outside of the cell membrane where presumably high-energy multicomponent reactions could not be utilized economically by the cell.

Since penicillin does not inhibit the staphylococcus *in vitro* system, it seemed clear that penicillin does not prevent the formation of the polysaccharide backbone. We were led to suspect, therefore, that penicillin was acting at the level of cross-linking since this appeared to be the only type of reaction not being measured in the penicillin-insensitive *in vitro* system for mucopeptide synthesis. If this were the mechanism, the mucopeptide being formed in the presence of penicillin would not be cross-linked and therefore should have a high content of free amino groups. If D-alanine

were ordinarily ejected during cross-linking, inhibition of this reaction by penicillin should yield a product with a higher content of alanine.

Test of the hypothesis: We have studied the formation of mucopeptide by staphylococci using conditions under which high concentrations of penicillin do not markedly inhibit formation of mucopeptide and have found the predicted result, namely, *the product formed in the presence of penicillin contained a markedly higher amount of alanine and a correspondingly higher number of free amino groups.*

As shown in Table 1, 20–40 per cent of the peptide side chains of the penicillin-inhibited cells had an extra alanine over and above that found in the control. It can be seen that bacitracin, which is believed to inhibit mucopeptide synthesis at the polysaccharide polymerization stage,¹⁷ does not have this effect, nor does tetracycline (which markedly inhibited residual protein synthesis in this experiment, data not shown). The relative content of lysine, indicated as excess lysine in Table 1, was not affected by penicillin or the other antibiotics. This basic experiment has been done many times with variations in the constituents of the medium or in the fractionation scheme with the results remaining essentially the same. The possibility of an obscure effect of penicillin on conversion of glucose to alanine seems remote. It should be noted that the content of alanine in the protein fraction from such experiments was also unchanged (data not shown). Similar results with penicillin were obtained under growth conditions. The theoretical maximum increase in alanine with complete inhibition of the cross-linking reaction relative to a completely linked product in the control is 100 per cent. We have obtained the

TABLE 1
EXCESS ALANINE AND INCREASED FREE GLYCINE AMINO GROUPS AS MEASURES OF
INTERFERENCE BY PENICILLIN WITH A CROSS-LINKING
TRANSEPTIDATION REACTION IN *S. aureus* H

Antibiotic added (μ g/ml)	Mucopeptide synthesized* (% of control)	Excess alanine, %†	Excess lysine, %‡	% of Terminal glycine amino groups free§
None	(100)	—	—	36
Penicillin, 0.2	76	20	<2	55
Penicillin, 1	61	25	<2	65
Penicillin, 10	52	42	<2	77
Bacitracin, 2	49	<2	—	40
Tetracycline, 10	100	<2	—	34

In this experiment bacteria were exposed to antibiotic for 20 min at 37° with shaking in a buffered 4 amino acid medium similar to that used previously.¹⁸ The incubation mixtures contained, in a final vol of 5 ml, 7.5 mg dry weight of washed, rapidly growing *S. aureus* H cells, 0.001 *M* L-alanine, 0.002 *M* glycine, 0.0004 *M* L-lysine, 0.002 *M* L-glutamic acid, 0.045 *M* glucose, radioactive amino acids at specific activities given below, antibiotics as listed above, and buffer and cofactors as described previously.¹⁸ Present in all flasks during this incubation were glycine-U-C¹⁴: 35,200 cpm per μ mole, and either alanine-U-H³: 232,000 cpm per μ mole, or lysine-U-H³: 347,000 cpm per μ mole. All incubations were performed in duplicate. After stopping the reaction with trichloroacetic acid, 5% final concentration, the mucopeptide and protein fractions were isolated essentially as previously.¹⁸

The uninhibited control values for incorporation of nuclide labels as cpm per 7.5 mg dry weight of cells per 20 min were glycine, 29,900; alanine, 50,700; and lysine, 41,700.

Free amino end groups were determined in the mucopeptide fraction following van Slyke nitrous acid treatment. Mucopeptide samples in 0.5 ml water were treated for 30 min at room temperature with 0.1 ml glacial acetic acid and 0.2 ml 8.4 *M* sodium nitrite. The tubes were then placed in ice, and 1.2 ml 4 *M* ammonium sulfamate was added slowly. The walls were hydrolyzed in 9 *N* H₂SO₄ at 121° in an autoclave for 2 hr, and the radioactive hydroxyacid was extracted by continuous ether extraction for 2 hr.

* Measured by glycine incorporation.

† This expression of the fraction of peptide side chains containing an extra alanine as compared with the control was calculated by dividing the alanine/glycine ratio in the experimental sample by the alanine/glycine ratio in the control, subtracting 1, and multiplying this result by 200, since there are normally 2 alanines in each peptide chain.

‡ This expression of the fraction of peptide side chains containing excess lysine as compared with the control was calculated by dividing the lysine/glycine ratio in the experimental sample by the lysine/glycine ratio in the control, subtracting 1, and multiplying by 100.

§ The cpm found in the ether extract of a nitrous acid-treated, hydrolyzed mucopeptide sample was divided by one fifth of the total glycine cpm of that sample, there being approximately 5 glycines per muramic acid in *S. aureus* H mucopeptide. The fraction was multiplied by 100 to give the per cent of terminal glycine amino groups free.

theoretical figure in some experiments lasting 15 min or more but not in shorter periods.

In *S. aureus*, a peptide composed of about 5 glycine residues is attached to the epsilon amino group of lysine prior to polymerization of the polysaccharide chain.¹⁶ In this case the transpeptidation reaction involves the free amino group of the terminal glycine residue. Table 1 shows that a much higher percentage of terminal glycine amino groups remained free in the mucopeptide synthesized in the presence of penicillin than in its absence. This indicates that much less cross-linking occurred in the presence of penicillin.

The extent of cross-linking in the newly synthesized mucopeptide of the control cells was 64 per cent in the experiment shown, since 36 per cent of the terminal glycine amino groups were found to be free. The extent of cross-linkage in the control varies somewhat with the age and condition of the cells used. However, the crucial penicillin effects, i.e., the excess of alanine over the control and the increase in terminal glycine free amino groups, were consistent findings. These two measures of the extent of cross-linkage roughly equal each other in the extent to which they deviate from the basal control values. There are errors in the methods which make it difficult to obtain an exact quantitative estimate of these two entities. Thus, the amount of glycine free amino groups found may be low, as the conversion of peptide-bound glycine to glycolic acid may not be quantitative.¹⁹ The amount of alanine found may be low because of dilution with newly synthesized nonradioactive alanine and because of possible loss of alanine caused by hydrolytic enzymes. We assume that these limitations apply equally to both the experimental and control cells so that the comparisons made in Table 1 are valid.

Discussion.—The data indicate that the cross-linking reaction in the synthesis of cell wall involves a transpeptidation which results in removal of the terminal D-alanine and that penicillin interferes with mucopeptide synthesis at this specific point. If this idea is borne out by further experiments with other organisms (as our preliminary results with *Bacillus megaterium* and *Escherichia coli* indicate), it would suggest that the same general type of cross-linking is vital to most bacteria, rickettsia, and viruses of the psittacosis-lymphogranuloma group since these are all sensitive to penicillin.

Other workers have also noted that high concentrations of penicillin sometimes do not inhibit net mucopeptide synthesis. Thus, Meadow²⁰ reported that an amount of penicillin which caused a marked drop in viability in 30 min did not reduce the rate of incorporation of diaminopimelic acid into the wall of *E. coli* during this time. Rogers and Jeljaszewicz²¹ have found that relatively high concentrations of penicillin for 10 or 15 min did not inhibit incorporation of C¹⁴-glutamic acid into the mucopeptide of *S. aureus* Oxford (under conditions similar to those we have used), although subsequent inhibition was marked.

The only previous work of which we are aware that might have detected the effect of penicillin on cross-linkage is that of Mandelstam and Rogers³ in which the effect of penicillin on the alanine content of *S. aureus* walls was reported. These authors found that the alanine content of the walls actually dropped in the presence of penicillin. This may be explained by the fact that they analyzed whole walls rather than isolated mucopeptide, and hence the alanine content of the penicillin-inhibited walls was the sum of the mucopeptide alanine which should have in-

creased plus the cell wall teichoic acid alanine which is known to be markedly decreased in the presence of penicillin.²²

Martin has demonstrated the existence and studied the composition of mucopeptide in certain stable and unstable L-forms of *Proteus mirabilis*.²³ In composition, this mucopeptide is similar to that of the parent bacillus. On the basis of his observation that the unstable L-form was producing mucopeptide and yet the wall could not protect the protoplast, Martin proposed that "Here, the penicillin seems to act more specifically, presumably by preventing the formation of certain cross-linkages within the mucopolymer which are indispensable for the establishment of shape and mechanical stability."²³

The nature of the reaction inhibited by penicillin would explain several observations difficult to understand in the past: (1) The ultimate destruction of a cell by penicillin would be the result of continued production of defective (non-cross-linked) mucopeptide which weakens the wall sufficiently to destroy membrane function. This damage would occur very rapidly in Gram negative cells. The loss of integrity of the mucopeptide may be accelerated by the action of lytic enzymes which attack mucopeptide^{24, 25} although there is no need for such "help" in cells which continue to produce defective mucopeptide since the wall would soon become defective under growth conditions.

(2) Of the several antibiotics which interfere with mucopeptide synthesis and cause accumulation of the precursor, UDP-acetyl muramyl pentapeptide, i.e., penicillin, vancomycin, bacitracin, and ristocetin,²⁶⁻²⁹ penicillin is the least effective in blocking net synthesis and in causing accumulation of precursors. The continued synthesis of incomplete or non-cross-linked mucopeptide in the presence of penicillin but not in the presence of the other antibiotics would explain this. Anderson *et al.*¹⁷ have recently shown that vancomycin, bacitracin, and ristocetin do inhibit the polysaccharide polymerization step in mucopeptide synthesis, whereas penicillin does not. (The cause of this inhibition is open to some question as vancomycin and bacitracin also inhibit growth of protoplasts,^{30, 31} and one would not expect specific inhibition of mucopeptide synthesis to be involved here.)

(3) A transpeptidation reaction involving elimination of D-alanine may explain the observation that a variety of D-amino acids cause spheroplast formation.³² It is possible that many different D-amino acids can compete for a site on the cross-linking enzyme and interfere with its function.

Penicillin as an analogue of the substrate for the cross-linking reaction: The action of penicillin at a point involving cross-linkage between peptides refocuses attention on the unstable "L-cysteinyl-D-valyl bond" in the β -lactam ring of penicillin as a possible analogue of a portion of the peptide involved in the cross-linkage. The portion of the peptide we believe penicillin most resembles is the L-alanyl- γ -D-glutamyl part of the molecule. The free α -carboxyl group of D-glutamic acid and the free carboxyl group of the antibiotic occupy similar positions relative to the peptide bond between the L and D amino acids of the two substances. The carboxyl of the penicillin molecule is essential for its activity. Collins and Richmond³³ have suggested that penicillin resembles N-acetyl muramic acid, but a key part of their argument required that the muramic acid carboxyl be free. This is not the case in nature. On the other hand, the α -carboxyl group of D-glutamic acid in mucopeptide is usually free. Penicillin would present a rigid molecule shaped so as

to fit that part of the active site of the hypothetical transpeptidase which binds L-alanyl- γ -D-glutamyl of the substrate. When the reactive β -lactam ring of penicillin is in close contact with the enzyme, it would react specifically and covalently with the active site of this enzyme, thereby inactivating it.

Summary.—Evidence is presented which indicates that penicillin interferes with a cross-linking reaction in bacterial mucopeptide synthesis. This reaction is a transpeptidation linking the penultimate D-alanine to the free amino group of a neighboring peptide chain with the release of the terminal D-alanine.

This paper is dedicated to the memory of Professor Wolfhard Weidel whose premature death was a great loss to the field of cell wall chemistry and to science in general.

* Supported by grant AI 05090 from the Institute of Allergy and Infectious Diseases, USPHS.

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